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(54) Title: THERMOSTABLE ENZYME PROMOTING THE FIDELITY OF THERMOSTABLE DNA POLYMERASES- FOR IMPROVEMENT OF NUCLEIC ACID SYNTHESIS AND AMPLIFICATION IN VITRO

(57) Abstract: A purified thermostable enzyme is derived from the thermophilic archaebacterium *Archaeoglobus fulgidus*. The enzyme can be native or recombinant, is stable under PCR conditions and exhibits double strand specific exonuclease activity. It is a 3'-5' exonuclease and cleaves to produce 5'-mononucleotides. Thermostable exonucleases are useful in many recombinant DNA techniques, in combination with a thermostable DNA polymerase like *Tag* especially for nucleic acid amplification by the polymerase chain reaction (PCR).

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Thermostable enzyme promoting the fidelity of thermostable DNA polymerases – for improvement of nucleic acid synthesis and amplification *in vitro*

The present invention is related to the field of molecular biology, and more particular, to polynucleotide synthesis. The present invention also relates to a substantially pure thermostable exonuclease, the cloning and expression of a thermostable exonuclease III in *E.coli*, and its use in amplification reactions. The invention facilitates the high fidelity amplification of DNA under conditions which allow decontamination from carry over and the synthesis of long products. The invention may be used for a variety of industrial, medical and forensic purposes.

In vitro nucleic acid synthesis is routinely performed with DNA polymerases with or without additional polypeptides. DNA polymerases are a family of enzymes involved in DNA replication and repair. Extensive research has been conducted on the isolation of DNA polymerases from mesophilic microorganisms such as *E.coli*. See, for example, Bessman et al. (1957) *J. Biol. Chem.* 223:171-177, and Buttin and Kornberg, (1966) *J. Biol. Chem.* 241:5419-5427.

Research has also been conducted on the isolation and purification of DNA polymerases from thermophiles, such as *Thermus aquaticus*. Chien, A. et al., (1976) *J. Bacteriol.* 127:1550-1557 discloses the isolation and purification of a DNA polymerase with a temperature optimum of 80°C from *Thermus aquaticus* YT1 strain. United States Patent No. 4,889,818 discloses a purified thermostable DNA polymerase from *T. aquaticus*, Taq polymerase, having a molecular weight of about 86,000 to 90,000 daltons. In addition, European Patent Application 0 258 017 discloses Taq polymerase as the preferred enzyme for use in the PCR process.

Research has indicated that while Taq DNA polymerase has a 5'-3' polymerase-dependent exonuclease function, Taq DNA polymerase does not possess a 3'-5' exonuclease III function (Lawyer, F.C. et al., (1989) J. Biol. Chem., 264:6427-6437; Bernad A., et al. (1989) Cell 59:219). The 3'-5' exonuclease activity of DNA polymerases is commonly referred to as "proofreading activity". The 3'-5' exonuclease activity removes bases which are mismatched at the 3' end of a

primer-template duplex. The presence of 3′-5′ exonuclease activity may be advantageous as it leads to an increase in fidelity of replication of nucleic acid strands and to the elongation of prematurely terminated products. As *Taq* DNA polymerase is not able to remove mismatched primer ends it is prone to base incorporation errors, making its use in certain applications undesirable. For example, attempting to clone an amplified gene is problematic since any one copy of the gene may contain an error due to a random misincorporation event. Depending on the cycle in which that error occurs (e.g., in an early replication cycle), the entire DNA amplified could contain the erroneously incorporated base, thus, giving rise to a mutated gene product.

There are several thermostable DNA polymerases known in the art which exhibit 3'- 5'exonuclease activity, like B-type polymerases from thermophilic Archaebacteria which are used for high fidelity DNA amplification. Thermostable polymerases exhibiting 3'- 5'exonuclease activity may be isolated or cloned from Pyrococcus (Purified thermostable Pyrococcus furiosus DNA polymerase, Mathur E., Stratagene, WO 92/09689, US 5,545,552; Purified thermostable DNA polymerase from Pyrococcus species, Comb D. G. et al., New England Biolabs, Inc., EP 0 547 359; Organization and nucleotide sequence of the DNA polymerase gene from the archaeon Pyrococcus furiosus, Uemori T. et al. (1993) Nucl. Acids Res., 21:259-265.), from Pyrodictium spec. (Thermostable nucleic acid polymerase, Gelfand D. H., F. Hoffmann-La Roche AG, EP 0 624 641; Purified thermostable nucleic acid polymerase and DNA coding sequences from Pyrodictium species, Gelfand D. H., Hoffmann-La Roche Inc., US 5,491,086), from Thermococcus (e.g. Thermostable DNA polymerase from Thermococcus spec. TY, Niehaus F., et al. WO 97/35988; Purified Thermocccus barossii DNA polymerase, Luhm R. A., Pharmacia Biotech, Inc., WO 96/22389; DNA polymerase from Thermococcus barossii with intermediate exonuclease activity and better long term stability at high temperature, useful for DNA sequencing, PCR etc., Dhennezel O. B., Pharmacia Biotech Inc., WO 96/22389; A purified thermostable DNA polymerase from Thermococcus litoralis for use in DNA manipulations, Comb D. G., New England Biolabs, Inc., US 5,322,785, EP 0 455 430; Recombinant thermostable DNA polymerase from Archaebacteria, Comb D. G., New England Biolabs, Inc., US 5,352,778, EP 0 547 920, EP 0 701 000; New isolated thermostable DNA polymerase obtained from Thermococcus gorgonarius, Angerer B. et al. Boehringer Mannheim GmbH, WO 98/14590.

Another possibility of conferring PCR in the presence of a proofreading function is the use of a mixture of polymerase enzymes, one polymerase exhibiting such a proofreading activity. (e.g. Thermostable DNA polymerase with enhanced thermostability and enhanced length and efficiency of primer extension, Barnes W. M., US 5,436,149, EP 0 693 078; Novel polymerase

compositions and uses thereof, Sorge J. A., Stratagene, WO 95/16028). It is common practice to use a formulation of a thermostable DNA polymerase comprising a majority component of at least one thermostable DNA polymerase which lacks 3′-5′ exonuclease activity and a minority component exhibiting 3′- 5′ exonuclease activity e.g. *Taq* polymerase and *Pfu* DNA polymerase. In these mixtures the processivity is conferred by the pol I-type enzyme like *Taq* polymerase, the proofreading function by the thermostable B-type polymerase like *Pfu*. High fidelity DNA synthesis is one desirable parameter in nucleic acid amplification, another important feature is the possibility of decontamination.

The polymerase chain reaction can amplify a single molecule over a billionfold. Thus, even minuscule amounts of a contaminant can be amplified and lead to a false positive result. Such contaminants are often poducts from previous PCR amplifications (carry-over contamination). Therefore, researchers have developed methods to avoid such a contamination.

The procedure relies on substituting dUTP for TTP during PCR amplification to produce uracil-containing DNA (U-DNA). Treating subsequent PCR reaction mixtures with Uracil-DNA-Gly-cosylase (UNG) prior to PCR amplification the contaminating nucleic acid is degraded and not suitable for amplification. dUTP can be readily incorporated by polI-type thermostable polymerases but not B-type polymerases (G. Slupphaug, et al. (1993) *Anal. Biochem.* 211:164-169) Low incorporation of dUTP by B- type polymerases limits their use in laboratories where the same type of template is repeatedly analyzed by PCR amplification.

Thermostable DNA polymerases exhibiting 3′ - 5′exonuclease activity were also isolated from eubacterial strains like *Thermotoga* (Thermophilic DNA polymerases from *Thermotoga neapolitana*, Slater M. R. et al. Promega Corporation, WO 96/41014; Cloned DNA polymerases from *Thermotoga neapolitana* and mutants thereof, Hughes A. J. et al., Life Technologies, Inc. WO 96/10640; Purified thermostable nucleic acid polymerase enzyme from *Termotoga maritima*, Gelfand D. H. et al., CETUS Corporation, WO 92/03556) These enzymes have a strong 3′-5′exonuclease activity which is able to eliminate misincorporated or mismatched bases. A genetically engineered version of this enzyme is commercially available as ULTma, a DNA polymerase which can be used without additional polypeptides for the PCR process. This enzyme is able to remove misincorporated bases, incorporate dUTP, but the fidelity is for unknown reasons not higher than that of Taq polymerase (Accuracy of replication in the polymerase chain reaction. Diaz R. S. et al. *Braz. J. Med. Biol. Res.* (1998) 31: 1239-1242; PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases, Cline J. et al., *Nucleic Acids Res.* (1996) 24:3546-3551).

For high fidelity DNA synthesis another alternative to the use of B-type polymerases or mixtures containing them is the use of thermophilic DNA polymerase III holoenzyme, a complex of 18 polypeptide chains. These complexes are identical to the bacterial chromosomal replicases, comprising all the factors necessary to synthesize a DNA strand of several hundred kilobases or whole chromosomes. The 10 different subunits of this enzyme, some of which are present in multiple copies, can be produced by recombinant techniques, reconstituted and used for *in vitro* DNA synthesis. As a possible use of these complexes PCR amplification of nucleic acis of several thousand to hundreds of thousand base pairs is proposed. (Enzyme derived from thermophilic organisms that functions as a chromosomal replicase, and preparation and uses thereof, Yurieva O. et al., The Rockefeller University, WO 98/45452; Novel thermophilic polymerase III holoenzyme, McHenry C., ENZYCO Inc., WO 99/13060)

It was aimed according to this invention to develop a high fidelity PCR system which is preferably concomitantly able to incorporate dUTP. According to the present invention a thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity is provided whereas this enzyme enhances fidelity of an amplification process when added to a second enzyme exhibiting polymerase activity. The enzyme provided can excise mismatched primer ends to allow the second enzyme exhibiting polymerase activity as e.g. Taq polymerase to reassociate and to reassume elongation during a process of synthezising DNA. The inventive enzyme is able to cooperate as proofreading enzyme with a second enzyme exhibiting polymerase activity. The enzyme that was found to be suitable for this task is e.g. a thermostable exonuclease III. Preferred is an exonuclease III working from the 3' to 5' direction, cleaving 5' of the phosphate leaving 3' hydroxyl groups and ideally working on double stranded DNA only. The 3'- 5'exonuclease functions of DNA polymerases are active on double and single stranded DNA. The latter activity may lead to primer degradation, which is undesired in PCR assays. It is preferred that the enzyme is active at 70 °C to 80 °C, stable enough to survive the denaturation cycles and inactive at lower temperatures to leave the PCR products undegraded after completion of the PCR process. Enzymes exhibiting these features can be derived from thermophilic eubacteria or related enzymes from thermophilic archaea. Genomes of three thermostable archaebacteria are sequenced, Methanococcus jannaschii (Complete Genome Sequence of the Methanogenic Archaeon, Methanococcus jannaschii, Bult C.J. et al., (1996) Science 273: 1058-1072), Methanobacterium thermoautotrophicum (Complete genomic sequence of Methanobacterium thermoautotrophicum ΔH : Functional Analysis and Comparative Genomics, Smith D.R. et al., J. of Bacteriology

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(1997) 179: 7135-7155) and Archaeoglobus fulgidus (The complete genome sequence of the hyperthermophilic, sulfate-reducing archaeon Archaeoglobus fulgidus, Klenk H.-P. et al. (1997) Nature 390: 364-370).

In particular, there is provided a thermostable enzyme obtainable from Archaeoglobus fulgidus, which catalyzes the degradation of mismatched ends of primers or polynucleotides in the 3′ to 5′ direction in double stranded DNA. The gene encoding the thermostable exonuclease III obtainable from Archaeoglobus fulgidus (Afu) was cloned, expressed in E.coli and isolated. The enzyme is active under the incubation and temperature conditions used in PCR reactions. The enzyme supports DNA polymerases like Taq in performing DNA synthesis at low error rates and synthesis of products of more than 3 kb on genomic DNA – the upper range of products synthesized by Taq polymerase - in good yields with or without dUTP present in the reaction mixture. Preferably, 50-500 ng of the exonuclease III obtainable from Afu were used per 2,5 U of Taq polymerase in order to have an optimal PCR performance. More preferably is the use of 67 ng to 380 ng of the exonuclease III obtainable from Afu per 2,5 U of the Taq polymerase in the PCR reaction.

Thus, the inventive enzyme is able to cooperate as proofreading enzyme with *Taq* polymerase. The advantage of the use of the inventive enzyme in comparison to other enzymes is that the inventive enzyme is preferably active on double stranded DNA. The thermostable enzyme of this invention may be used for any purpose in which such enzyme activity is necessary or desired. In a particularly preferred embodiment the enzyme is used in combination with a thermostable DNA polymerase in the nucleic acid amplification reaction known as PCR in order to remove mismatched primer ends which lead to premature stops, to provide primer ends which are more effectively elongated by the polymerase, to correct for base incorporation errors and to enable the polymerase to produce long PCR products.

Further, subject of the present invention is a composition comprising a first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and a second enzyme exhibiting polymerase activity whereas the fidelity of an amplification process is enhanced by the use of this composition in comparison to the use of the second enzyme alone. The inventive thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity also includes appropriate enzymes exhibiting reduced DNA polymerase

activity or no such activity at all. Reduced DNA polymerase activity according to the invention means less than 50% of said activity of an enzyme exhibiting DNA polymerase activity. In a preferred embodiment the second enzyme of the inventive composition is lacking proofreading activity. In particular preferred, the second enzyme is Taq polymerase.

A further subject of the present invention is a method of DNA synthesis using a mixture comprising a first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and a second enzyme exhibiting polymerase activity. According to this method prematurely terminated chains are trimmed by degradation from 3' to 5'. Mismatched ends of either a primer or the growing strand are removed according to this method.

The invention further comprises a method according to the above description whereas dUTP is present in the reaction mixture, replacing partly or completely TTP. It is preferred that according to this method uracil DNA glycosylase (UDG or UNG) is used for degradation of contaminating nucleic acids.

Preferably. according to this method the mixture of a

- first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and
- a second enzyme exhibiting polymerase activity produces PCR products with lower error rates compared to PCR products produced by the second enzyme exhibiting polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity. The method in which the mixture of first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and a second enzyme exhibiting polymerase activity produces PCR products of greater length compared to PCR products produced by the second enzyme exhibiting polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity. Further, the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity is related to the Exonuclease III of E. coli, but thermostable according to this method. A further embodiment of the above described method is the method whereas PCR products with blunt ends are obtained.

Subject of the present invention are also methods for obtaining the inventive thermostable enzyme exhibiting 3' exonuclease-activity but essentially no DNA polymerase activity and means

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and materials for producing this enzyme as e.g. vectors and host cells (e.g. DSM no. 13021).

The following examples are offered for the purpose of illustrating, not limiting, the subject invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Brief description of the drawings

Figure 1:

DNA sequence and the deduced amino acid sequence of the gene encoding the DNA polymerase from exonuclease III of *Archaeoglobus fulgidus*.

Figure 2:

Resistance to heat denaturation of the recombinant exonuclease III of Archaeoglobus fulgidus expressed in E.coli as described in Example V.

Lane 1: Incubation at 50°C

Lane 2: Incubation at 60°C

Lane 3: Incubation at 70°C

Lane 4: Incubation at 80°C

Lane 5: Incubation at 90°C

Lane 6: E.coli host cell extract not transformed with gene encoding Afu exonuclease III

Lane 7: Exonuclease III of E.coli

Lane 8: Molecular weight marker

Figure 3:

Exonuclease activity of Afu exonuclease III on DNA fragments as described in Example VI.

Lane 1: 10 units E.coli exonuclease III, incubation at 37°C

Lane 2: 50 ng of Afu exonuclease III, incubation at 72°C

Lane 3: 100 ng of Afu exonuclease III, incubation at 72°C

Lane 4: 150 ng of Afu exonuclease III, incubation at 72°C

Lane 5: 100 ng of Afu exonuclease III, incubation at 72°C

Lane 6: 200 ng of Afu exonuclease III, incubation at 72°C

Lane 7: 300 ng of Afu exonuclease III, incubation at 72°C

Lane 8: 250 ng of Afu exonuclease III, incubation at 72°C

Lane 9: 750 ng of Afu exonuclease III, incubation at 72°C

Lane 10: 1 µg of Afu exonuclease III, incubation at 72°C

Lane 11: 500 ng of Afu exonuclease III, incubation at 72°C

Lane 12: 1 µg of Afu exonuclease III, incubation at 72°C

Lane 13: 1.5 µg of Afu exonuclease III, incubation at 72°C

Lane 14: 1.5 µg of Afu exonuclease III, incubation at 72°C

Lane 15: 3 µg of Afu exonuclease III, incubation at 72°C

Lane 16: 4.5 µg of Afu exonuclease III, incubation at 72°C

Lane 17: 7.6 µg of Afu exonuclease III, incubation at 72°C

Lane 18: 15.2 µg of Afu exonuclease III, incubation at 72°C

Lane 19: 22.8 µg of Afu exonuclease III, incubation at 72°C

Lane 20: no exonuclease added

Figure 4:

Principle of the mismatch correction assay.

Figure 5:

Mismatched primer correction in PCR as described in Example VII.

- Lane 1: DNA Molecular Weight Marker V (ROCHE Molecular Biochemicals No. 821705)
- Lane 2: G:A mismatched primer, amplification with Taq DNA polymerase
- Lane 3: same as in lane 2, but subsequently cleaved with BsiEI
- Lane 4: G:A mismatched primer, amplification with Expand HiFi PCR System
- Lane 5: same as in lane 4, but subsequently cleaved with BsiEI
- Lane 6: G:A mismatched primer, amplification with Taq polymerase/Afu exonuclease III
- Lane 7: same as in lane 6, but subsequently cleaved with BsiEI
- Lane 8: G:A mismatched primer, amplification with Tgo DNA polymerase
- Lane 9: same as in lane 8, but subsequently cleaved with BsiEI
- Lane 10: G:T mismatched primer, amplification with Tag DNA polymerase
- Lane 11: same as in lane 10, but subsequently cleaved with BsiEI
- Lane 12: G:T mismatched primer, amplification with Expand HiFi PCR System
- Lane 13: same as in lane 12, but subsequently cleaved with BsiEI
- Lane 14: G:T mismatched primer, amplification with Tag polymerase/Afu exonuclease III
- Lane 15: same as in lane 14, but subsequently cleaved with BsiEI
- Lane 16: G:T mismatched primer, amplification with Tgo DNA polymerase
- Lane 17: same as in lane 16, but subsequently cleaved with BsiEI
- Lane 18: DNA Molecular Weight Marker V
- Lane 19: DNA Molecular Weight Marker V

- Lane 20: G:C mismatched primer, amplification with Tag DNA polymerase
- Lane 21: same as in lane 20, but subsequently cleaved with BsiEI
- Lane 22: G:C mismatched primer, amplification with Expand HiFi PCR System
- Lane23: same as in lane 22, but subsequently cleaved with BsiEI
- Lane 24: G:C mismatched primer, amplification with Taq polymerase/Afu exonuclease III
- Lane 25: same as in lane 24, but subsequently cleaved with BsiEI
- Lane 26: G:C mismatched primer, amplification with Tgo DNA polymerase
- Lane 27: same as in lane 26, but subsequently cleaved with BsiEI
- Lane 28: CG:AT mismatched primer, Taq DNA polymerase
- Lane 29: same as in lane 28, but subsequently cleaved with BsiEI
- Lane 30: CG:AT mismatched primer, Expand HiFi PCR System
- Lane 31: same as in lane 2, but subsequently cleaved with BsiEI
- Lane32: CG:AT mismatched primer, Taq polymerase/Afu exonuclease III
- Lane 33: same as in lane 2, but subsequently cleaved with BsiEILane 34: CG:AT mismatched
- primer, amplification with Tgo DNA polymerase
- Lane 35: same as in lane 2, but subsequently cleaved with BsiEI
- Lane 36: DNA Molecular Weight Marker V.

Figure 6A:

Error rates of different polymerases in PCR

Figure 6B:

Improvement of fidelity by Afu exonuclease III present in the PCR mixture as described in Example VIII.

The ratio of blue:white colonies were blottet and various mixtures of *Taq* DNA polymerase and *Afu* exonuclease III (Taq/Exo 1:30, Taq/Exo 1:20, Taq/Exo 1:15, Taq/Exo 1:12,5, Taq/Exo 1:10 corresponding to 2.5 units of *Taq* DNA polymerase mixed with 125 ng, 175 ng, 250 ng, 375 ng and 500 ng of *Afu* exonuclease III, respectively) were tested in comparison to *Taq* DNA polymerase (Taq), Expand HiFi PCR System (HiFi) and *Pwo* DNA polymerase (Pwo).

Figure 7:

Incorporation of dUTP by the Taq DNA polymerase / Afu exonuclease III mixture as described in Example IX.

- Lane 1: DNA Molecular Weight Marker XIV (Roche Molecular Biochemicals No. 1721933)
- Lane 2: Amplification with 2.5 units Tag DNA polymerase

- Lane 3: Amplification with 2.5 units Taq DNA polymerase and 125 ng of Afu exonuclease III
- Lane 4: Amplification with 2.5 units Taq DNA polymerase and 250 ng of Afu exonuclease III
- Lane 5: Amplification with 2.5 units Taq DNA polymerase and 375 ng of Afu exonuclease III
- Lane 6: Amplification with 2.5 units Taq DNA polymerase and 500 ng of Afu exonuclease III

Figure 8:

Degradation of dUTP containing PCR products by Uracil-DNA Glycosylase as described in Example IX.

- Lane 1: DNA Molecular Weight Marker XIV (Roche Molecular Biochemicals No. 1721933)
- Lane 2: 1 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
- Lane 3: 2 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
- Lane 4: 3 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
- Lane 5: 4 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
- Lane 6: 5 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III and subsequent UNG and heat treatment.
- Lane 7: 5 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III no subsequent UNG or heat treatment.
- Lane 8: 5 µl of the amplification product obtained with *Taq* DNA polymerase and 125 ng of *Afu* exonuclease III no subsequent UNG but heat treatment.
- Lane 9: DNA Molecular Weight Marker XIV (Roche Molecular Biochemicals No. 1721933)

Figure 9:

Effect of Afu exonuclease III on PCR product length. The Taq DNA polymerase / Afu exonuclease III mixture was analyzed on human genomic DNA as described in Example X.

Lane 1:9,3 kb tPA fragment with Taq/Exo III Mix

Lane 2: " Taq-Pol.

Lane 3: 12 kb tPA fragment with Taq/Exo III Mix

Lane 4: "Taq-Pol.

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Lane 5: 15 kb tPA fragment with Taq/Exo III Mix

Lane 6: "Taq-Pol.

Figure 10:

Thermostable exonuclease III can be replaced by a polymerase mutant with reduced polymerase activity but increased 3'-exonucleoase-activity as described in Example XI.

Lane 1: Molecular Weight Marker

Lane 2: reaction 1, Taq polymerase, 4.8 kb fragment

Lane 3: reaction 2, Taq polymerase plus Tag polymerase mutant, 4.8 kb fragment

Lane 4: reaction 3, no Taq polymerase, Tag polymerase mutant, 4.8 kb fragment

Lane 5: reaction 4, Taq polymerase plus Afu ExoIII, 4.8 kb fragment

Lane 6: reaction 5, Taq polymerase, 9.3 kb fragment

Lane 7: reaction 6, Taq polymerase plus Tag polymerase mutant, 9.3 kb fragment Lane 8:

reaction 7, no Taq polymerase, Tag polymerase mutant, 9.3 kb fragment

Lane 9: reaction 8, Taq polymerase plus Afu ExoIII, 9.3 kb fragment

Lane 10: Molecular Weight Marker

Figure 11.

Afu exonuclease III is not active on linear single stranded DNA as described in Example XII

Lane 1: Afu Exo III, no incubation

Lane 2: Afu Exo III, 1 h at 65°C

Lane 3: Afu Exo III, 2 h at 65°C

Lane 4: Afu Exo III, 3 h at 65°C

Lane 5: Afu Exo III, 4 h at 65°C

Lane 6::Afu Exo III, 5 h at 65°C

Lane 7: Reaction buffer without enzyme, no incubation

Lane 8: Reaction buffer without enzyme, 5 h at 65°C

Lane 9: Molecular Weight Marker

Figure 12:

Comparison of Afu exonuclease III with a thermostable B-type polymerase in primer degradating activity as described in Example XIII.

Molecular Weight Marker Lane 1: 1 u Tgo preincubated (reaction 1) Lane 2: Lane 3: 1.5 u Tgo, preincubated (reaction 2) 1 u Tgo, not preincubated (reaction 3) Lane 4: Lane 5: 1.5 u Tgo, not preincubated (reaction 4) Lane 6: 1 u Tgo, preincubated in the absence of dNTPs (reaction 5) Lane 7: 1.5 u Tgo, preincubated in the absence of dNTPs (reaction 6) 1 u Tgo, not preincubated in the absence of dNTPs (reaction 7) Lane 8: Lane 9: 1.5 u Tgo, not preincubated in the absence of dNTPs (reaction 8) Lane 10: 1 u Tgo, preincubated, in the absence of dNTPs, supplemented with additional primer (reaction 9) Lane 11: 1.5 u Tgo, preincubated in the absence of dNTPs, supplemented with additional primer (reaction 10) Lane 12: Taq polymerase, preincubated (reaction 11) Lane 13: Taq plus 37,5 ng Afu Exo III, preincubated (reaction 12) Lane 14: Taq plus 75 ng Afu Exo III, preincubated (reaction 13) Lane 15: Taq polymerase, not preincubated (reaction 14) Lane 16: Taq plus 37,5 ng Afu Exo III, not preincubated (reaction 15) Lane 17: Taq plus 75 ng Afu Exo III, not preincubated (reaction 16) Lane 18: Molecular Weight Marker

EXAMPLE 1

Isolation of coding sequences

The preferred thermostable enzyme herein is an extremely thermostable exodeoxyribonuclease obtainable from *Archaeoglobus fulgidus* VC-16 strain (DSM No. 4304). The strain was isolated from marine hydrothermal systems at Vulcano island and Stufe di Nerone, Naples, Italy (Stetter, K. O. et al., *Science* (1987) 236:822-824). This organism is an extremely thermophilic, sulfur metabolizing, archaebacteria, with a growth range between 60°C and 95°C with optimum at 83°C. (Klenk, H.P. et al., *Nature* (1997) 390:364-370). The genome sequence is deposited in the TIGR data base. The gene putatively encoding exonuclease III (xthA) has Acc.No. AF0580.

The apparent molecular weight of the exodeoxyribonuclease obtainable from *Archaeoglobus* fulgidus is about 32,000 daltons when compared with protein standards of known molecular weight (SDS-PAGE). The exact molecular weight of the thermostable enzyme of the present invention may be determined from the coding sequence of the *Archaeoglobus fulgidus* exodeoxyribonuclease III gene.

EXAMPLE II

Cloning of the gene encoding exonuclease III from Archaeoglobus fulgidus

About 6 ml cell culture of DSM No. 4304 were used for isolation of chromosomal DNA from *Archaeoglobus fulgidus*.

The following primers were designed with restriction sites compatible to the multiple cloning site of the desired expression vector and complementary to the N- and C-terminus of the *Archaeo-globus fulgidus* exonuclease III gene:

SEQ ID NO.: 1

N-terminus (BamHI-site): 5'-GAA ACG AGG ATC CAT GCT CAA AAT CGC CAC C -3'

SEQ ID NO.: 2

C-terminus (PstI-site): 5'-TTG TTC ACT GCA GCT ACA CGT CAA ACA CAG C -3'

First the cells were collected by repeted centrifugation in one 2 ml eppendorf cap at 5,000 rpm. The DNA isolation may be performed with any described method for isolation from bacterial cells. In this case the *Archaeoglobus fulgidus* genomic DNA was prepared with the High PureTM PCR Template Preparation Kit (ROCHE Diagnostics GmbH, No. 1796828). With this method about 6 µg chromosomal DNA were obtained with a concentration of 72 ng/µl.

PCR was performed with the primers described above, in the Expand™ High Fidelity PCR System (ROCHE Diagnostics GmbH, No. 1732641) and 100 ng Archaeoglobus fulgidus genomic DNA per cap in four identical preparations. PCR was performed with the following conditions:

- 1 x 94°C, 2 min;
- 10 x 94°C, 10 sec; 54°C, 30 sec; 68°C, 3 min;

- 20 x 94°C, 10 sec; 54°C, 30 sec; 68°C, 3 min with 20sec cycle elongation for each cycle;
- 1 x 68°C, 7 min;

After adding MgCl₂ to a final concentration of 10 mM the PCR product was cleaved with BamHI and Pst I, 10 units each, at 37°C for 2 hours. The reaction products were separated on a low-melting agarose gel. After electrophoresis the appropriate bands were cut out, the gel slices combined, molten, the DNA fragments isolated by agarase digestion and precipitated with EtOH. The dried pellet was diluted in $30 \,\mu$ l H₂O.

The appropriate expression vector, here pDS56_T, was digested with the same restriction enzymes as used for the insert and cleaned with the same method.

After ligation of insert and vector with the Rapid DNA Ligation Kit (ROCHE Diagnostics GmbH, No.1635379) the plasmid was transformed in the expression host *E.coli* 392 pUBS520 (Brinkmann, U. et al. (1989) *Gene* 85:109-114).

Plasmid DNA of the transformants was isolated using the High Pure™ Plasmid Isolation Kit (ROCHE Diagnostics GmbH, No.1754777) and characterized by restriction digestion with BamHI and PstI and agarose gel electrophoresis.

Positive E.coli pUBS520 ExoIII transformants were stored in glycerol culture at -70°C. The sequence of the gene encoding exonuclease III was confirmed by DNA sequencing. It is shown in Figure No. 1.

Cloning and expression of exonuclease III from *Archaeoglobus fulgidus* or other thermophilic organisms may also be performed by other techniques using conventional skill in the art (see for example Sambrook et al. Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Lab., 1989).

EXAMPLE III

Expression of recombinant Afu exonuclease III

The transformant from example I was cultivated in a fermentor in a rich medium containing appropriate antibiotic. Cells were harvested at an optical density of [A₅₄₀] 5.5 by centrifugation

and frozen until needed or lyzed by treatment with lysozyme to produce a crude cell extract containing the *Archaeoglobus fulgidus* exonuclease III activity.

The crude extract containing the *Archaeoglobus fulgidus* exonuclease III activity is purified by the method described in example IV, or by other purification techniques such as affinity-chromatography, ion-exchange-chromatography or hydrophobic-interaction-chromatography.

EXAMPLE IV

Purification of recombinant Afu exonuclease III

E.coli pUBS520 ExoIII (DSM No. 13021) from example I was grown in a 101 fermentor in media containing tryptone (20 g/l), yeast extract (10 g/l), NaCl (5 g/l) and ampicillin (100 mg/l) at 37°C, induced with IPTG (0.3 mM) at midexponential growth phase and incubated an additional 4 hours. About 45 g of cells were harvested by centrifugation and stored at – 70°C. 2 g of cells were thawed and suspended in 4 ml buffer A (40 mM Tris/HCl, pH 7.5; 0.1 mM EDTA; 7 mM 2-mercaptoethanol; 1mM Pefabloc SC). The cells were lyzed under stirring by addition of 1.2 mg lysozyme for 30 minutes at 4°C and addition of 4.56 mg sodium deoxycholate for 10 minutes at room temperature followed by 20 minutes at 0°C. The crude extract was adjusted to 750 mM KCl, heated for 15 minutes at 72°C and centrifuged for removal of denatured protein.

A heating temperature up to 90 °C is also possible without destroying (denaturation) the Archaeoglobus fulgidus exonuclease III. The supernatant was dialyzed against buffer B (buffer A containig 10 % glycerol) adjusted to 10 mM MgCl₂ and applied to a Blue Trisacryl M column (SERVA, No. 67031) with the dimension 1 x 7 cm and 5.5 ml bed volume, equilibrated with buffer B. The column was washed with 16.5 ml buffer B and the exonuclease protein was eluted with a 82 ml linear gradient of 0 to 3 M NaCl in buffer B. The column fractions were assayed for Archaeoglobus fulgidus exodeoxyribonuclease protein by electrophoresis on 10-15% SDS-PAGE gradient gels. The active fractions, 16.5 ml, were pooled, concentrated with Aquacide II (Calbiochem No. 17851) and dialyzed against the storage buffer C (10 mM Tris/HCl, pH 7.9; 10 mM 2-mercptoethanol; 0.1mM EDTA; 50 mM KCl; 50 % glycerol). After dialysis Thesit and Nonidet P40 were added to a final concentration of 0.5% each. This preparation was stored at - 20 °C.

The Archaeoglobus fulgidus exonuclease III obtained was pure to 95% as estimated by SDS gel electrophoresis. The yield was 50 mg of protein per 2.3g cellmass (wetweight).

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EXAMPLE V

Thermostability of recombinant exonuclease III from Archaeoglobus fulgidus

The thermostability of the exonuclease III from *Archaeoglobus fulgidus* cloned as described in Example II was determined by analyzing the resistance to heat denaturation. After lysis as described in Example IV 100 µl of the crude extract were centrifuged at 15,000 rpm for 10 min in an Eppendorf centrifuge. The supernatant was aliquoted into five new Eppendorf caps. The caps were incubated for 10 minutes at five different temperatures, 50°C, 60°C, 70°C, 80°C and 90°C. After centrifugation as described above, aliquotes of the supernatants were analyzed by electrophoresis on 10-15 % SDS-PAGE gradient gels. As shown in Figure 2 the amount of *Archaeoglobus fulgidus* exonuclease III protein after incubation at 90°C was the same as that of the samples treated at lower temperatures. The was no significant loss by heat denaturation detectable. From this result it can be concluded that the half life is more than ten minutes at 90°C.

EXAMPLE VI

Activity of Afu exonuclease III

Exonuclease III catalyzes the stepwise removal of mononucleotides from 3'-hydroxyl termini of duplex DNA (Rogers G.S. and Weiss B. (1980) *Methods Enzymol.* 65:201-211). A limited number of nucleotides are removed during each binding event. The preferred substrate are blunt or recessed 3'-termini. The enzyme is not active on single stranded DNA, and 3'-protruding termini are more resistant to cleavage. The DNA Molecular Weight Marker VI (ROCHE Molecular Biochemicals, No.1062590) consists of BgII digested pBR328 mixed with HinfI digested pBR328. The products of the HinfI digest have 3'-recessive termini and are expected to be preferred substrates to degradation by exonuclease III, the products of BgII cleavage have 3'protruding ends with 3 bases overhangs and should be more resistant to cleavage by exonuclease III.

Serial dilutions of *Archaeoglobus fulgidus* exonuclease III from Example IV were incubated for 2 hours at 72 °C with 0.5 µg DNA Molecular Weight Marker VI (ROCHE Molecular Biochemicals, No.1062590) in 25 µl of the following incubation buffer: 10 mM Tris/HCl, pH 8.0; 5 mM MgCl₂; 1 mM 2-mercaptoethanol; 100 mM NaCl with Paraffin overlay. 10 units of exonuclease III of *E.coli* (ROCHE Molecular Biochemicals, No.779709) was included as a control. The control

reaction was performed at 37°C. After addition of 5 µl stop solution (0.2 % Agarose, 60 mM EDTA, 10 mM Tris-HCl, pH 7.8, 10 % Glycerol, 0.01 % Bromphenolblue) the mixtures were separated on a 1 % agarose gel. The result is shown in Figure 3. Afu exonuclease III discriminates between the two different types of substrate. The preferred substrate are the fragments with 3′-recessive ends (e.g. 1766 bp fragment) and the 3′-overhanging ends (e.g. 2176 bp, 1230bp, 1033 bp fragments) are more resistant to degradation. With higher amounts of protein the substrate is degraded to a similar extent as in lane 1, where the products of exonuclease III of E.coli were analyzed. With increasing amounts of Afu exonuclease protein only little DNA substrate was left (lanes 15 to 19), the retardation of the remaining fragments may be due to DNA binding proteins as impurities of the preparation.

EXAMPLE VII

Mismatched primer correction in PCR with Afu exonuclease III

The repair efficiency of the Afu exonuclease III / Taq polymerase mixture during PCR was tested with 3' terminally mismatched primers, the principle of the assay is shown in Figure 4. For PCR amplification sets of primers are used in which the forward primer has one or two nucleotides at the 3' end which cannot base pair with the template DNA. Excision of the mismatched primer end and amplification of the repaired primer generates a product which can subsequently be cleaved with the restriction endonuclease BsiEI, whereas the product arising from the mismatched primer is resistant to cleavage.

The primer sequences used:

5. forward 4 (2 base mismatch):

reverse: 5' - GGT TAT CGA AAT CAG CCA CAG CG - 3' (SEQ ID NO.: 3)
 forward 1 (g:a mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TCA - 3' (SEQ ID NO.: 4)
 forward 2 (g:t mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TCT - 3' (SEQ ID NO.: 5)
 forward 3 (g:c mismatch): 5' - TGG ATA CGT CTG AAC TGG TCA CGG TCC - 3' (SEQ ID NO.: 6)

5' - TGG ATA CGT CTG AAC TGG TCA CGG TAT - 3'

(SEQ ID NO.: 7)

PCR was carried out using 2.5 Units Taq DNA Polymerase (ROCHE Diagnostics GmbH, No. 1435094), 0.25 µg of Archaeoglobus fulgidus exonuclease III from Example IV, 10 ng of DNA from bacteriophage λ , 0.4 µM of each primer, 200 µM of dNTP's, 1.5 mM of MgCl₂, 50 mM of Tris-HCl, pH 9.2, 16 mM of (NH₄)₂SO₄. PCR was performed in an volume of 50µl PCR with the following conditions:

- 1 x 94°C, 2 min;
- 40 x 94°C, 10 sec; 60°C, 30 sec; 72°C, 1 min;
- 1 x 72°C, 7 min;

The function of the exonuclease/*Taq* polymerase mixture was compared to controls as 2.5 Units of *Taq* DNA polymerase, 0.3 Units of *Tgo* DNA polymerase (ROCHE Diagnostics GmbH) and to 0.75 μl of ExpandTM High Fidelity PCR System (ROCHE Diagnostics GmbH, No.1732641). As indicated by successful digestion of the PCR products with BsiEI *A. fulgidus* exonuclease III showed correcting activity of all described mismatches with an effectivity of 90 to 100 % (Figure 5). *Taq* DNA Polymerase as expected showed no correcting activity, while *Tgo* DNA Polymerase with it's 3′-5′ exonuclease activity corrected completely as well. The ExpandTM High Fidelity PCR System showed only with the two base mismatch 100% correcting activity. The other mismatches were repaired with an effectivity of approximately 50%.

EXAMPLE VIII

Fidelity of Afu exonuclease III /Taq DNA polymerase mixtures in the PCR process

The fidelity of Afu exonuclease III/Taq DNA polymerase mixtures in the PCR process was determined in an assay based on the amplification, circularisation and transformation of the pUC19 derivate pUCIQ17, containing a functional lac I^q allele (Frey, B. and Suppmann B. (1995) Biochemica 2:34-35). PCR-derived mutations in lac I are resulting in a derepression of the expression of lac Z\alpha and subsequent formation of a functional \(\beta\)-galactosidase enzyme which can be easily detected on X-Gal indicator plates. The error rates of Taq polymerase \(Afu\) exonuclease mixtures determined with this \(lac\) I-based PCR fidelity assay were determined in comparison to \(Taq\) DNA polymerase and Expand HiFi PCR System (Roche Molecular Biochemicals) and \(Pwo\) DNA polymerase (Roche Molecular Biochemicals) as controls.

The plasmid pUCIQ17 was linearized by digestion with DraII to serve as a substrate for PCR amplification with the enzymes tested.

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Both of the primers used have ClaI sites at their 5 prime ends:

SEQ ID NO.: 8

Primer 1: 5'-AGCTTATCGATGGCACTTTTCGGGGAAATGTGCG-3'

SEQ ID NO.: 9

Primer 2: 5'-AGCTTATCGATAAGCGGATGCCGGGAGCAGACAAGC-3'

The length of the resulting PCR product is 3493 bp.

The PCR was performed in a final volume of 50 μ l in the presence of 1.5 mM MgCl₂, 50 mM Tris⁻ HCl, pH 8.5 (25°C), 12.5 mM (NH₄)₂SO₄, 35 mM KCl, 200 μ M dNTPs and 2.5 units of *Taq* polymerase and 125 ng, 175 ng, 250 ng, 375 ng and 500 ng, respectively of *Afu* exonuclease III.

The cycle conditions were as follows:

1 x denaturation of template for 2 min. at 95°C

denaturation at 95°C for 10 sec.
annealing at 57°C for 30 sec.
elongation at 72°C for 4 min.

denaturation at 95°C for 10 sec.

annealing at 57°C for 30 sec.

elongation at 72°C for 4 min.

+ cycle elongation of 20 sec. for each cycle

After PCR, the PCR products were PEG-precipitated (Barnes, W. M. (1992) Gene 112:229) the DNA restricted with ClaI and purified by agarose gel electrophoresis. The isolated DNA was ligated using the Rapid DNA Ligation Kit (Roche Molecular Biochemicals) and the ligation products transformed in *E.coli* DH5α, plated on TN Amp X-Gal plates. The α-complementing *E.coli* strain DH5α transformed with the resulting plasmid pUCIQ17 (3632 bp), shows white (lacI⁺) colonies on TN plates (1.5 % Bacto Tryptone, 1 % NaCl, 1.5 % Agar) containing ampicillin (100 μg/ml) and X-Gal (0.004 % w/v). Mutations result in blue colonies.

After incubation overnight at 37°C, blue and white colonies were counted. The error rate (f) per bp was calculated with a rearranged equation as published by Keohavong and Thilly (Keohavong, P. and Thilly, W. (1989) *PNAS* USA 86:9253):

$$f = -\ln F / dxbbp$$

where F is the fraction of white colonies:

F =white (lacI+) colonies / total colony number;

d is the number of DNA duplications:

and b is the effective target size of the (1080bp) *lac* I gene, which is 349 bp according to Provost et al. (Provost et al. (1993) *Mut. Res.* 288:133).

The results shown in Figure 6A and Figure 6B demonstrate that the presence of thermostable exonuclease III in the reaction mixure results in lower error rates. Dependent on the ratio of polymerase to exonuclease the error rate is decreasing. The fidelity achieved with the most optimal Taq polymerase / Afu exonuclease III mixture (4,44 x 10⁻⁶) is in a similar range as that of the Taq/Pwo mixture (Expand HiFi; 2,06 x 10⁻⁶). Evaluation of the optimal buffer conditions will further improve the fidelity. The ratio between polymerase and exonuclease has to be optimized. High amounts of exonuclease reduce product yield, apparently decreasing amplification efficiency (Taq/Exo 1:10 corresponding to 2.5 units of Taq polymerase and 500 ng of Afu exonuclease III).

The fidelity of this system may further be optimized using conventional skill in the art e.g. by altering the buffer components, optimizing the concentration of the individual components or changing the cycle conditions.

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EXAMPLE IX:

Incorporation of dUTP in the presence of Afu exonuclease III during PCR

The Afu exonuclease /Taq polymerase mixture was tested for DNA synthesis with TTP completely replaced by dUTP. Comparisation of either TTP or dUTP incorporation was determinated in PCR using 2.5 Units of Taq DNA Polymerase, in presence of 0.125 µg, 0.25 µg, 0.375 µg and 0.5 µg of Archaeoglobus fulgidus exonuclease III from example IV on native human genomic DNA as template using the ß-globin gene as target. The following primers were used:

forward: 5' - TGG TTG AAT TCA TAT ATC TTA GAG GGA GGG C - 3'

(SEQ ID NO.: 10)

reverse: 5' - TGT GTC TGC AGA AAA CAT CAA GGG TCC CAT A - 3'

(SEQ ID NO.: 11)

PCR was performed in 50 µl volume with the following cycle conditions:

1 x 94°C, 2 min;

40 x 94°C, 10 sec; 60°C, 30 sec; 72°C, 1 min;

1 x 72°C, 7 min;

Aliquots of the PCR reaction were separated on agarose gels. As shown in Figure 7 with this template/primer system DNA synthesis in the presence of dUTP is possible with up to 375 ng of Afu exonuclease III. dUTP incorporation can further be proven by Uracil-DNA Glycosylase treatment (ROCHE Diagnostics GmbH, No.1775367) of aliquotes from the PCR reaction products for 30 min at ambient temperature and subsequent incubation for 5 min at 95°C to cleave the polynucleotides at the apurinic sites which leads to complete degradation of the fragments. The analysis of the reaction products by agarose gel electrophoresis is shown in Figure 8.

EXAMPLE X:

Effect of Afu exonuclease III on PCR product length

Taq polymerase is able to synthesize PCR products up to 3 kb in length on genomic templates. In order to estimate the capability of the Taq polymerase/Afu exonuclease mixture for the synthesis of longer products, the enzyme mixture was analyzed on human genomic DNA as template with

three pairs of primers designed to amplify products of 9.3 kb, 12 kb and 15 kb length. The buffer systems used were from the Expand Long Template PCR System (Roche Molecular Biochemicals Cat. No 1 681 834). Reactions were performed in 50 μ l volume with 250 ng of human genomic DNA, 220 ng of each primer, 350 μ M of dNTPs and 2.5 units of Taq polymerase and 62,5 ng of Afu exonuclease with the conditions as outlined in Table 1:

Table 1:

Product length	Primers	Expand Long Template buffer No.:	PCR Programm
9.3 kb	forward 7 reverse 14	1	1 x denat. at 94 °C for 2 min 10 x denat. at 94 °C for 10 sec. annealing at 65 °C for 30 sec elogation at 68 °C for 8 min. 20 x denat. at 94 °C for 10 sec. annealing at 65 °C for 30 sec elogation at 68 °C for 8 min. plus cycle elongation of 20 sec. per cycle
•			1 x elongation at 68°C for 7 min.
12 kb	forward 1 reverse 3	2	1 x denat. at 94 °C for 2 min 10 x denat. at 94 °C for 10 sec. annealing at 62 °C for 30 sec elogation at 68 °C for 12 min. 20 x denat. at 94 °C for 10 sec. annealing at 62 °C for 30 sec elogation at 68 °C for 12 min. plus cycle elongation of 20 sec. per cycle 1 x elongation at 68 °C for 7 min.
15 kb	forward 1 reverse 2	3	same as for 12 kb

The primer specific for amplification of the tPA genes used:

Primer 7a forward: 5' - GGA AGT ACA GCT CAG AGT TCT GCA GCA CCC CTG C - 3'

(SEQ ID NO.: 12)

Primer 14a reverse: 5'- CAA AGT CAT GCG GCC ATC GTT CAG ACA CAC C-3'

(SEQ ID NO.: 13)

Primer 1 forward: 5'- CCT TCA CTG TCT GCC TAA CTC CTT CGT GTG TCC C-3'

(SEQ ID NO.: 14)

Primer 2 reverse: 5' - ACT GTG CTT CCT GAC CCA TGG CAG AAG CGC CTT C-3'

(SEQ ID NO.: 15)

Primer 3 reverse: 5'- CCT TCT AGA GTC AAC TCT AGA TGT GGA CTT AGA G - 3'

(SEQ ID NO.: 16)

As shown in Figure 9 it is possible to synthesize products of at least 15 kb in length with the *Taq* polymerase/*Afu* exonuclease mixture.

Example XI

Thermostable Exonuclease III can be replaced by a polymerase mutant with reduced polymerase activity but increased 3' exonuclease-activity

DNA polymerase from Thermococcuss aggregans (Tag) described from Niehaus F., Frey B. and Antranikian G. in WO97/35988 or *Gene* (1997) 204 (1-2), 153-8, with an amino acid exchange at position 385 in which tyrosine was replaced by asparagine (Boehlke at al. submitted for publication and European patent application 00105 155.6) shows only 6.4 % of the polymerase activity but 205 % of the exonuclease activity of the wild type DNA polymerase. This enzyme was used to demonstrate that the invention is not restricted to exonuclease III-type enzymes but also includes other types of enzymes contributing 3' exonuclease activity.

Reactions were performed in 50 µl volume with 200 ng of human genomic DNA, 200 µM dNTP, 220 ng of each primer and Expand HiFi buffer incl. Mg⁺⁺ for reactions 1-4 or Expand Long Template buffer 1 for reactions 5-8 (Figure 10). In order to amplify a 4.8 kb fragment of the tPA gene, primer tPA 7a forward (5′-GGA AGT ACA GCT CAG AGT TCT GCA GCA CCC CTG C-3′, SEQ ID NO.: 12) and tPA 10a reverse (5′-GAT GCG AAA CTG AGG CTG GCT GTA CTG TCT C-3′, SEQ ID NO.: 17) were used in reactions 1 – 4. In order to amplify a 9.3 kb fragment of of the tPA gene, primer tPA 7a forward and tPA 14a reverse (5′-CAA AGT CAT GCG GCC ATC GTT CAG ACA CAC C-3′, SEQ ID NO.: 13) were used in reactions 5-8. 2.5 units Taq polymerase were added to reactions 1,2,4,5,6, and 8, not to reactions 3 and 7 which were used as negative controls. 11 ng of Tag polymerase mutant were added to reactions 2,3, 6 and 7, 150 ng of Afu Exonuclease III were added to reactions 4 and 8.

The cycle programs used for reactions 1-4:

```
1 x 94°C, 2 min,

10 x 94°C, 10 sec

62°C, 30 sec

68°C, 4 min

20 x 94°C, 10 sec

62°C, 30 sec
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68°C, 4 min, plus cycle elongation of 20 sec per cycle 1x 68°C for 7 min

for reactions 5-8:

```
1 x 94°C, 2 min,

10 x 94°C, 10 sec

65°C, 30 sec

68°C, 8 min

20 x 94°C, 10 sec

65°C, 30 sec

68°C, 8 min, plus cycle elongation of 20 sec per cycle

1x 68°C for 7 min
```

The PCR products were analysed on a 1 % agarose gel containg ethidium bromide (Figure 10). The data show that Taq polymerase is able to amplify the 4.8 kb fragment but with low yield. The combination of Taq polymerase with Tag polymerase mutant or Afu Exo III results in a strong increase in product yield. The Tag polymerase mutant enzyme by itself is not able to synthesize this product.

Similar results were obtained with the 9.3 kb system. Using Taq polymerase alone no product is detectable. In combination with Tag polymerase mutant or Afu Exo III the expected PCR product is obtained in high yield.

These results show that Taq polymerase is not able to amplify DNA fragments of several kb from genomic DNA and support the hypothesis of Barnes (Barnes W. M. (1994) *Proc. Natl. Acad. Sci.* USA, 91:2216-2220) that the length limitation for PCR amplification is caused by low efficiency of extension at the sites of incorporation of mismatched base pairs. After removal of the mismatched nucleotide at the primer end, Taq polymerase is able to reassume DNA synthesis. The completed nucleic acid chain as a full length product can then serve as a template for primer binding in subsequent cycles.

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Example XII

Afu Exo III is not active on linear single stranded DNA

Reactions were performed in 50 μ l volume with 270 ng of Afu Exo III, 5 μ g of a 49-mer oligonucleotide in Expand HiFi PCR buffer with MgCl₂ and incubated for 0, 1, 2, 3, 4, and 5 hours at 65°C. After addition of 10 μ l of Proteinase K solution (20 mg/ml) the samples were incubated for 20 min. at 37°C. The reaction products were analysed on a 3.5 % Agarose gel containing ethidium bromide.

The result is depicted in figure 11. It showes that the nucleic acid has the same size in all lanes. The product obtained after incubation for up to 5 hours (lane 6) with Afu Exo III has the same size as the controls (lanes 1, 7 and 8). Neither a significant reduction in intensity of the full length oligonucleotide nor a smear deriving from degraded products can be observed.

Example XIII

Comparison of Afu Exonuclease III with a thermostable B-type polymerase in primer degradating activity

Thermostable B-type polymerases are reported to have single and double stranded nuclease activity (Kong H. et al. (1993) *Journal Biol. Chem.* 268:1965-1975). This activity is able to degrade primer molecules irrespective whether they are hybridized to the template or single stranded. The replacement of a thermostable B-type polymerase by a thermostable exonuclease in the reaction mixture might be of advantage with respect to stability of single stranded primer or other nuclei acids present in the reaction mixture.

In order to test for primer degrading activity, reaction mixtures without template DNA were incubated for 1 hour at 72°C, then DNA was added and PCR was performed. The results were compared with reactions containing Tgo polymerase as an example for a thermostable B-type polymerase (Angerer B. et al. WO 98/14590). As control the same mixtures were used without prior incubation. The results are summarized in Table 2.

Table 2:

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reaction #	enzyme (s)	preincubation in the absence of template DNA	preinc. in the presence of nucleotides	second addition of primer after preincubaion
1	Tgo	yes	yes	
2	Tgo	yes	yes	
3	Tgo	no		
4	Tgo	no		
5	Tgo	yes	no	
6	Tgo	yes	no	
7	Tgo	no		
8	Tgo	no		
9	Tgo	yes	no	yes
10	Tgo	yes	no	yes
11	Taq	yes	yes	
12	Taq plus Afu Exo III	yes	yes	
13	Taq plus Afu Exo III	yes	yes	
14	Taq	no		
15	Taq plus Afu Exo III	no		
16	Taq plus Afu Exo III	no		

As target for amplification a fragment of the p53 gene was chosen, the primer used were: p53I 5′-GTC CCA AGC AAT GGA TGA T-3′ (SEQ ID NO.: 18) and p53II 5′-TGG AAA CTT TCC ACT TGA T-3′ (SEQ ID NO.: 19). PCR reactions were performed in 50 µl volume.

Reactions nos. 1 – 10 contained 200 ng of human genomic DNA, 40 pmole of each primer, 10 mM Tis-HCl, pH 8.5, 17.5 mM (NH₄)₂SO₄, 1.25 mM MgCl₂, 0.5 % Tween, 2.5 % DMSO, 250 μ g/ml BSA and 1 unit (reactions number 1, 3, 5, 7 and 9) or 1.5 units (reactions number 2, 4, 6, 8 and 10) Tgo polymerase and 200 μ M dNTPs.

Reactions number 11 to 16 contained 2.5 units Taq polymerase, Expand HiFi buffer with Mg $^{++}$, 40 pmoles of primer, 200 μ M dNTPs, 100 ng human genomic DNA. Reactions number 12 and 15 contained 37.5 ng of Afu Exo III, reactions number 13 and 16 contained 75 ng of Afu Exo III.

As described in table 2 reactions 1, 2, 5, 6 and 11 to 13 were incubated for 1 hour at 72°C in the absence of template DNA. The template DNA was added before PCR was started. Reactions 5, 6, 9 and 10 were preincubated in the absence of nucleotides, reactions 9 and 10 were supplemented with additional 40 pmoles of primer after the preincubation step. Because of the 5′- exonuclease activity of Taq polymerase, the enzyme was added after preincubation to reactions 11 to 13.

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PCR conditions:

1 x 94°C, 2 min

35 x 94°C, 10 sec 55°C, 30 sec 72°C, 4 min

1x 72°C for 10 min

The reaction products were analysed on an agarose gel and stained with ethidium bromide (Figure 12).

When Tgo polymerase was incubated with the primer in the absence of template DNA (reactions 1,2,5 and 6) and compared with the corresponding reactions without preincubation (3,4,7 and 8) a clear difference was observed. The preincubation results in strongly reduced PCR product obviously affecting at least one essential component, most probably the PCR primer. Extra addition of 40 pmoles of PCR primer (reactions 9 and 10) after the preincubation step results in strong signals with intensities comparable to the control reaction which were not preincubated. This shows that Tgo polymerase, a thermostable B-type polymerase, degrades PCR primer in the absence of template no matter whether dNTPs are present or not.

The PCR products obtained with reactions 12 and 13, in which the primer were preincubated with Afu Exonuclease III before addition of template DNA and Taq polymerase gave similar bands as those obtained with reactions 15 and 16, in which no preincubation step was used. From the similar strong band intensities it can be concluded that little or no degradation of primer occurred and that single stranded oligonucleotides are poor substrates for Afu Exonuclease III. From the strong band intensities or enhanced yields of PCR products it can be concluded that the enzyme enhances fidelity of an amplification process.

CLAIMS:

- Thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase
 activity whereas this enzyme enhances fidelity of an amplification process when added to a
 second enzyme exhibiting polymerase activity.
- 2. Thermostable enzyme according to claim 1 obtainable from Archeoglobus fulgidus.
- 3. Thermostable enzyme according to claim 1 or 2 whereas this enzyme is able to cooperate as proofreading enzyme with a second enzyme exhibiting polymerase activity.
- 4. Thermostable enzyme according to claim 1, 2 or 3 whereas the enzyme exhibits reduced DNA polymerase activity.
- 5. Composition comprising a first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and a second enzyme exhibiting DNA polymerase activity whereas the fidelity of an amplification process is enhanced by the use of the composition in comparison to the use of the single second enzyme.
- 6. Composition according to claim 5 whereas the second enzyme is lacking proofreading activity.
- 7. Composition according to claim 5 or 6 whereas the second enzyme is Taq polymerase.
- 8. A method of preparing or amplifying DNA using a composition according to claim 6 or 7.
- 9. The method of claim 8 whereas prematurely terminated chains are trimmed by degradation from 3' to 5'.
- 10. The method according to one of the claims 8 or 9 whereas mismatched ends of either a primer or the growing strand are removed.
- 11. The method according to one of the claims 8 to 10 whereas dUTP instead of TTP is present in the reaction mixture.

- 12. The method according to claim 11 whereas UNG is used for degradation of contaminating nucleic acids.
- 13. The method according to one of the claims 8 to 12 whereas the mixture of a
 - first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and
 - a second enzyme exhibiting DNA polymerase activity produces PCR products with lower error rates compared to PCR products produced by the second enzyme exhibiting DNA polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity.
- 14. The method of claim 13 in which the mixture of first thermostable enzyme exhibiting 3'exonuclease-activity but essentially no DNA polymerase activity and a second enzyme
 exhibiting DNA polymerase activity produces PCR products of greater length compared to
 PCR products produced by the second enzyme exhibiting DNA polymerase activity in
 absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no
 DNA polymerase activity
- 15. The method according to one of the claims 8 to 14 whereas the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity is related to the Exonuclease III derived from E.coli, but is thermostable.
- 16. The method according to one of the claims 8 to 15 whereas PCR products with blunt ends are obtained.
- 17. A method for amplifying DNA using a thermostable enzyme exhibiting 3'-exonuclease-activity which enzyme is not or only to a negligible extend active on linear single stranded DNA.
- 18. The method according to claim 17 wherein an enzyme according to any of claims 1 to 4 is used.

Figure 1: 1/2

Sequence of the Archaeoglobus fulgidus exonuclease III gene:

SEQ ID NO.: 20/21																										
	1	ATGCTCAAAATCGCCACCTTCAACGTAAACTCCATCAGGAGCAGACTGCACATCGTGATT 1++++++														60										
		TAC	CGA	GTT:	ATT	GCG(GTG	GAA	GTT.	GCA	TTT	GAG	GTA	GTC	CTC	GTC	TGA	CGT	GTA	GCA	CTAA					
a		M	L	K	I	A	T	F	Ŋ	V	N	s	I	R	S	R	L	Н	I	V	I	-				
																					GAAC					
	61																				CTTG	120				
a		P	W	L	K	E	N	K	P	D	I	L	С	М	Q	E	T	ĸ	v	E	N	_				
		AG	AGGAAGTTTCCTGAGGCCGATTTTCACCGCATCGGCTACCACGTCGTCTTCAGCGGGAGC																							
	121		TCCTTCAAAGGACTCCGGCTAAAAGTGGCGTAGCCGATGGTGCAGCAGAAGTCGCCCTCG															180								
a		R	ĸ	F	P	F.	Α	D	F	н	R	Т	G	Υ	н	V	v	F	s	G	S	_				
ű												_		_							_					
	181	AAGGGAAGGAATGGAGTGGCCATAGCTTCCCTCGAAGAGCCTGAGGATGTC 181						240																		
			_																							
a		K	G	R	N	G	V	Α	I	A	S	L	Е	E	P	E	D	V	S	F	G	-				
	241	CTCGATTCAGAGCCGAAGGACGAGGACAGGCTGATAAGGGCAAAGA												300												
		GA	GCT	AAG:	CTC	CGG	CTT	CCT	GCT	CCT	GTC	CGA	CTA	TTC	CCG	TTT	CTA	TCG.	ACC	GTA	TCTG					
a		L	D	S	E	P	K	D	E	D	R	L	I	R	A	K	I	A	G	I	D	-				
	201		GTGATTAACACCTACGTTCCTCAGGGATTCAAAATTGACAGCGAGAAGTACCAGTACAAG													260										
	301																				GTTC	360				
a		V	I	N	T	Y	V	P	Q	G	F	K	I	D	s	E	K	Y	Q	Y	K	-				
		CT	CCA	GTG	GCT'	rga(GAG	GCT	TTA	.CCA	TTA	CCT	TCA	AAA	AAC	CGT	TGA	СТТ	CAG	AAG	CTTT					
	361	GAGGTCACCGAACTCTCCGAAATGGTAATGGAAGTTTTTTGGCAACTGAAGTCTTCGAAA														420										
a		L	Q	W	L	E	R	L	Y	Н	Y	L	Q	K	т	V	D	F	R	s	F	_				
		GC'	TGT'	TTG	GTG!	rgg/	AGA	CAT	'GAA	.CGT	TGC	TCC	TGA	.GCC	AAT	CGA	CGT	TCA	CTC	CCC	AGAC					
	421																				TCTG	480				
a				W																						
a																										
	481				-+-			+				+			-+-			+			ACTC +	540				
		TT(CGA	CTT(CTT	GGT(GCA	GAC	GAA	GGT	GCT	CCT	ACG	CTC	TTC	CCG	TAT	GTT	TTT	TTA	TGAG					
а		K	L	K	N	Н	V	С	F	Н	E	D	A	R	R	A	Y	K	K	I	L	-				

Figure 1: 2/2

	E # 1		AACTCGGCTTTGTTGACGTGCTGAGAAAATACATCCCAACGAGAGAATTTACACCTTC															600				
	541		CTTGAGCCGAAACAACTGCACGACTCTTTTTATGTAGGGTTGCTCTCTTAAATGTGGAAG															600				
a		E	L	G	F	v	D	V	L	R	K	I	Н	P	N	E	R	I	Y	T	F	-
	TACGACTACAGGGTTAAGGGAGCCATTGAGCGGGGGCTGGGATGGAGGGTTGAT 601+											+										
a		Y	D	Y	R	v	К	G	A	I	E	R	G	L	G	W	R	v	D	Α	I	-
	661																				GAGG	720
		GAGCGGTGGGGTGGGAGCGCTTTCTACGCACCTGACGATGCGTCTGTAGTTTGGCTCC																				
a		L	A	T	P	P	L	A	E	R	С	V	D	С	Y	A	D	I	K	P	R	-
	CTGGCAGAAAAGCCATCCGACCACCTCCCTCTCGTTGCTGTTTTGACGT																					
	721		CCG'																		74	
a		L	А	E	ĸ	P	s	D	Н	· L	P	L.	v	Α	V	F	D	v	*	_		

Figure 2:

Temperature stability of Afu exonuclease III

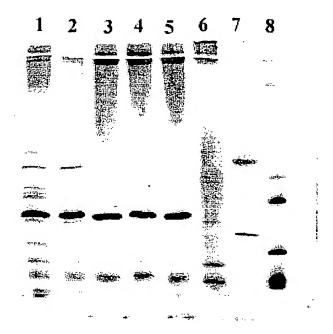


Figure 3:
Test for exonuclease III activity

1 2 3 4 5 6 7 8 9 10 11 12 13 14 .

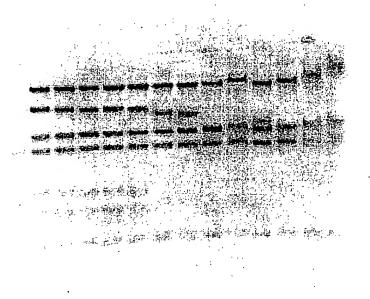


Figure 4:

Principle of the 3'-primer correction assay

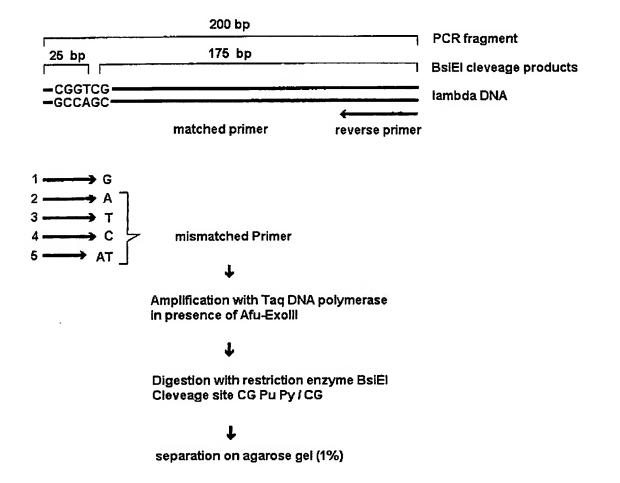


Figure 5:
Mismatched primer correction in PCR

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18





19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

Figure 6A

Error rates of different DNA polymerases in PCR

5991 5.4 5.4 5908 0.72 5908 0.29 7790 1.6 7315 1.08 2724 0.92	Polymerase	Template	yield	DNA duplica-	blue colonies	white	total	% lac	error rate
Ch. 10 11650 10.2 130 2261 2391 5.4 Ch. 10 11550 10.2 40 5458 5498 0.72 Exo 1 10 9675 9.9 17 5891 5908 0.29 Exo 1 10 11550 10.2 94 4291 4385 2.14 Exo 2 10 11125 10.1 146 7644 7790 1.87 Exo 3 10 8500 9.7 133 8188 8321 1.6 Exo 4 10 1292 7 79 7356 7315 1.08 Exo 5 10 256 2674 2724 0.92 2724 0.92		conc. (ng)	(Bu)	tions d	laci	lac	of colonies		(f ₃₄₉₎
Ch. 10 11550 10.2 40 5458 5498 0.72 Exo 1 10 9675 9.9 17 5891 5908 0.29 Exo 1 10 11550 10.2 94 4291 4385 2.14 Exo 2 10 11125 10.1 146 7644 7790 1.87 Exo 3 10 8500 9.7 133 8188 8321 1.6 Exo 4 10 1292 7 79 7236 7315 1.08 Exo 5 10 236 4.6 25 2574 2724 0.92	rag Ch.	10	11650	10.2	130	2261	2391	5.4	1.57 × 10°
EXO 1 10 9675 9.9 17 5891 5908 0.29 EXO 1 10 11550 10.2 94 4291 4385 2.14 EXO 2 10 11125 10.1 146 7644 7790 1.87 EXO 3 10 8500 9.7 133 8188 8321 1.6 EXO 4 10 1292 7 79 7236 7315 1.08 EXO 5 10 236 4.6 25 2674 2724 0.92	IFI Ch.	10	11550	10.2	40	5458	5498	0.72	2.06 x 10 ⁻⁵
10 11550 10.2 94 4291 4385 2.14 10 11125 10.1 146 7644 7790 1.87 10 8500 9.7 133 8188 8321 1.6 10 1292 7 79 7236 7315 1.08 10 236 4.6 25 2674 2724 0.92)wo	10	9675	6.6	17	5891	2908	0.29	8.32 × 10'
10 11125 10.1 146 7644 7790 1.87 10 8500 9.7 133 8188 8321 1.6 10 1292 7 79 7236 7315 1.08 10 236 4.6 25 2674 2724 0.92	aq/Exo 1	10	11550	10.2	94	4291	4385	2.14	6.10 × 10 ⁻⁵
10 8500 9.7 133 8188 8321 1.6 10 1292 7 79 7236 7315 1.08 10 236 4.6 25 2674 2724 0.92	aq/Exo 2	10	11125	10.1	146	7644	7790	1.87	5.36 x 10°
10 1292 7 79 7236 7315 1.08 10 236 4.6 25 2674 2724 0.92	aq/Exo 3	10	8500	9.7	133	8188	8321	1.6	4.74 × 10 ⁻⁶
10 236 4.6 25 2674 2724 0.92	aq/Exo 4	10	1292	7	79	7236	7315	1.08	4.44 × 10°
	aq/Exo 5	10	236	4.6	25	2674	2724	0.92	1.16 x 10°C

* Due to the unfavorable ratio of Taq:Exo the product yield was low. This results in an apparently low amplification efficiency d, which is an important parameter in the formula used for the calculation of the error rate.

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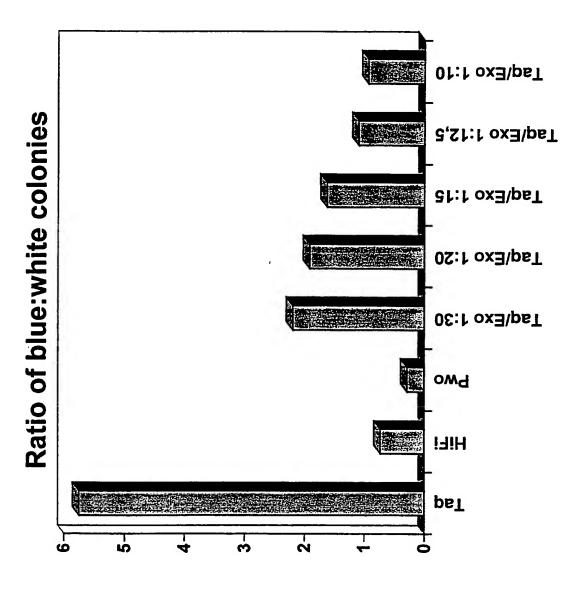
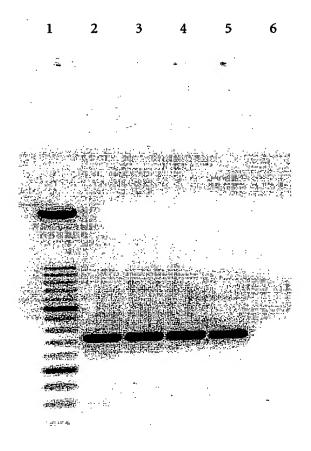


Figure 6B:

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Figure 7:



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Figure 8:

UNG treatment of dUMP containing PCR products

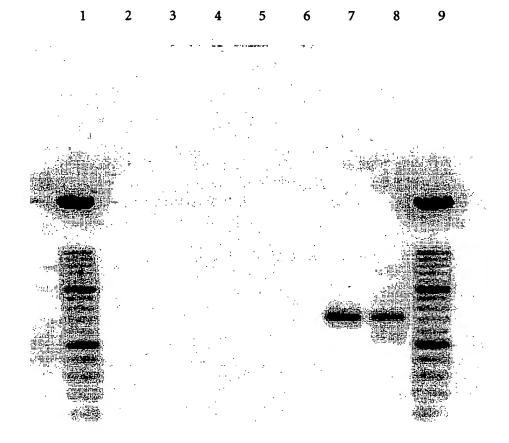


Figure 9:

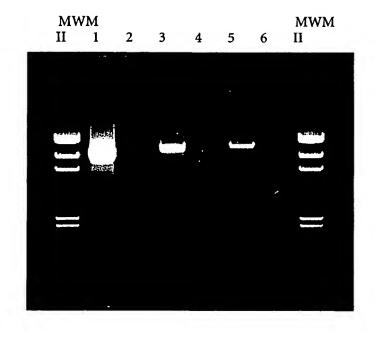


Figure 10:

1	2	3	4	5	6	7	8	9	10
MWM	1	2	3	4	5	6	7	8	MWM

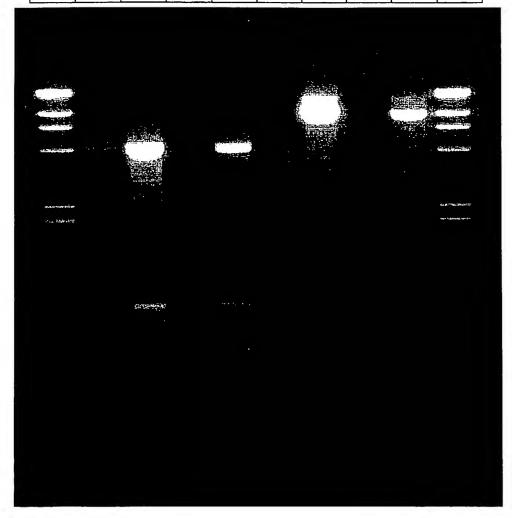


Figure 11

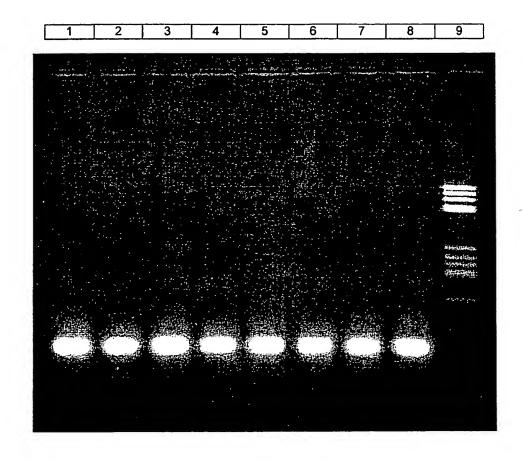
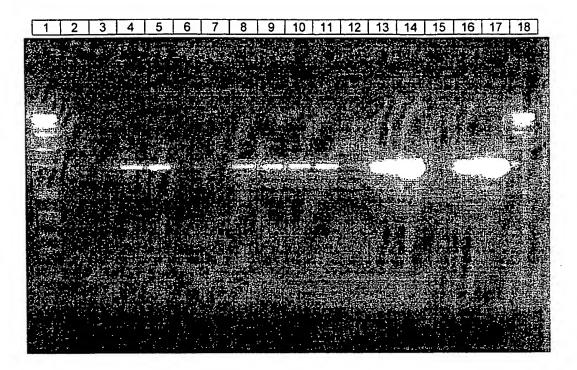


Figure 12



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atg cag gag acg aag gtt gag aac agg aag ttt cct gag gcc gat ttt Met Gln Glu Thr Lys Val Glu Asn Arg Lys Phe Pro Glu Ala Asp Phe 35 40 45	144
cac cgc atc ggc tac cac gtc gtc ttc agc ggg agc aag gga agg aat His Arg Ile Gly Tyr His Val Val Phe Ser Gly Ser Lys Gly Arg Asn 50 55 60	192
gga gtg gcc ata gct tcc ctc gaa gag cct gag gat gtc agc ttc ggt Gly Val Ala Ile Ala Ser Leu Glu Glu Pro Glu Asp Val Ser Phe Gly 65 70 75 80	240
ctc gat tca gag ccg aag gac gag gac agg ctg ata agg gca aag ata Leu Asp Ser Glu Pro Lys Asp Glu Asp Arg Leu Ile Arg Ala Lys Ile 85 90 95	238
gct ggc ata gac gtg att aac acc tac gtt cct cag gga ttc aaa att Ala Gly Ile Asp Val Ile Asn Thr Tyr Val Pro Gln Gly Phe Lys Ile 100 105 110	336
gac agc gag aag tac cag tac aag ctc cag tgg ctt gag agg ctt tac Asp Ser Glu Lys Tyr Gln Tyr Lys Leu Gln Trp Leu Glu Arg Leu Tyr 115 120 125	384
cat tac ctt caa aaa acc gtt gac ttc aga agc ttt gct gtt tgg tgt His Tyr Leu Gln Lys Thr Val Asp Phe Arg Ser Phe Ala Val Trp Cys 130 135 140	432
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aaa aaa ata ctc gaa ctc ggc ttt gtt gac gtg ctg aga aaa ata cat Lys Lys Ile Leu Glu Leu Gly Phe Val Asp Val Leu Arg Lys Ile His 180 185 190	576
ccc aac gag aga att tac acc ttc tac gac tac agg gtt aag gga gcc Pro Asn Glu Arg Ile Tyr Thr Phe Tyr Asp Tyr Arg Val Lys Gly Ala 195 200 205	624
att gag cgg ggg ctg gga tgg agg gtt gat gcc atc ctc gcc acc cca Ile Glu Arg Gly Leu Gly Trp Arg Val Asp Ala Ile Leu Ala Thr Pro 210 215 220	672
ccc ctc gcc gaa aga tgc gtg gac tgc tac gca gac atc aaa ccg agg Pro Leu Ala Glu Arg Cys Val Asp Cys Tyr Ala Asp Ile Lys Pro Arg 225 230 235 240	720
ctg gca gaa aag cca tcc gac cac ctc cct ctc gtt gct gtg ttt gac Leu Ala Glu Lys Pro Ser Asp His Leu Pro Leu Val Ala Val Phe Asp 245 250 255	768
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- (74) Common Representative: ROCHE DIAGNOSTICS GMBH; Patent Department, 68298 Mannheim (DE).
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(75) Inventors/Applicants (for US only): ANKENBAUER, Waltraud [DE/DE]; Oberanger 18, 82377 Penzberg (DE). For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: THERMOSTABLE ENZYME PROMOTING THE FIDELITY OF THERMOSTABLE DNA POLYMERASES- FOR IMPROVEMENT OF NUCLEIC ACID SYNTHESIS AND AMPLIFICATION IN VITRO

Error rates of different DNA polymerases in PCR

Polymerase	Template	yield	DNA duplica-	blue colonies	white colonies	total number	% lac	error rate
	conc. (ng)	(ng)	tions d	tacī	lac*	of colonies		(fore)
raq Ch.	10	11650	10.2	130	2261	2391	5.4	1.57 x 10°
			<u> </u>					
Hifi Ch.	10	11550	10.2	40	5458	5498	0.72	2.06 x 10°
	40	9675	9.9	17	5891	5908	0.29	8.32 x 10°
Pero	10	30/3	5.5					
Tan/Exo 1	10	11550	10.2	94	4291	4385	2.14	6.10 x 10
Tag/Exo 2	10	11125	10.1	146	7644	7790	1.87	5.38 x 10*
Tag/Exo 3	10	8500	9.7	133	8188	8321	1.6	4.74 x 10*
Yeq/Exo 4	10	1292	7	79	7236	7315	1.08	4.44 x 10°
Taq/Exo 5	10	238	4.6	25	2674	2724	0.92	1.16 x 10 ⁻¹⁷

^{*} Due to the unfavorable ratio of Taq:Exo the product yield was low. This results in an apparently low amplification efficiency d, which is an important parameter in the formula used for the calculation of the error rate.

(57) Abstract: A purified thermostable enzyme is derived from the thermophilic archaebacterium *Archaeoglobus fulgidus*. The enzyme can be native or recombinant, is stable under PCR conditions and exhibits double strand specific exonuclease activity. It is a 3'-5' exonuclease and cleaves to produce 5'-mononucleotides. Thermostable exonucleases are useful in many recombinant DNA techniques, in combination with a thermostable DNA polymerase like *Tag* especially for nucleic acid amplification by the polymerase chain reaction (PCR).



1/23583 A3

Internation pplication No PCT/EP 00/09423

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/55 C12N15/54 C12N15/10 C12N9/22 C12N9/12 C12Q1/68 C12P19/34 According to International Patent Classification (IPC) or to both national classification and IPC B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12N C12Q C12P IPC 7 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practical, search terms used) WPI Data, PAJ, CAB Data, STRAND, EPO-Internal C. DOCUMENTS CONSIDERED TO BE RELEVANT Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No. Category * H.P. KLENK ET AL.: "Archaeoglobus 1-3 X fulgidus section 43 of 172 of the complete genome" **EMBL SEQUENCE DATABASE** 1 December 1997 (1997-12-01), XP002139308 Hinxton, UK cited in the application 17,18 Y Accession no. AE001064, TrEMBLrel 029675, 01-01-1998; EXODEOXYRIBONUCLEASE III (XTHA): --/--Patent family members are tisted in annex. Further documents are listed in the continuation of box C. Special categories of cited documents: "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the "A" document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled *O* document referring to an oral disclosure, use, exhibition or in the art. document published prior to the international filing date but later than the priority date claimed '&' document member of the same patent family Date of mailing of the international search report Date of the actual completion of the international search **77**7, 05, 01 10 May 2001 Authorized officer Name and mailing address of the ISA

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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo ni.

Hornig, H

Internation. pplication No PCT/EP 00/09423

		FC1/EF 00/03423
C.(Continu	BUON) DOCUMENTS CONSIDERED TO BE RELEVANT	
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	cited in the application Locus AF0580, exodeoxyribonuclease III (xthA);	17,18
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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

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Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)
This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)
This International Searching Authority found multiple inventions in this international application, as follows:
see additional sheet
As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable dalms could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Remark on Protest The additional search fees were accompanied by the applicant's protest. X No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: (1-3,5-16)-complete, 18-partially

Thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity whereas this enzyme enhances fidelity of an amplification process when added to a second enzyme exhibiting polymerase activity; said enzyme obtainable from Archaeglobus fulgidus, whereas this enzyme is able to cooperate as proofreading enzyme with a second enzyme exhibiting polymerase activity; Composition comprising a first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity and a second enzyme exhibiting DNA polymerase activity whereas the fidelity of an amplification process is enhynced by the use of the composition to the use of the single second enzyme; a method of preparing or amplifying DNA using said composition; said method whereas the mixture of a -first thermostable enzyme exhibiting 3'-exonucleaseactivity but essentially no DNA polymerase activity and -a second enzyme exhibiting DNA polymerase activity produces PCR products with lower error rates compared to PCR products produced by the second enzyme exhibiting DNA polymerase activity in absence of the first thermostable enzyme exhibiting 3'-exonuclease-activity but essentially no DNA polymerase activity; said method whereas PCR products with blunt ends are obtained; a method for amplifying DNA using said thermostable enzyme exhibiting 3'-exonuclease-activity which enzyme is not or only to a negligible extend active on linear single stranded DNA;

2. Claims: 4-complete, 18-partially

Thermostable enzyme exhibiting 3'-exonuclease-activity but exhibits reduced DNA polymerase activity; a method for amplifying DNA using said thermostable enzyme exhibiting 3'-exonuclease-activity which enzyme is not or only to a negligible extend active on linear single stranded DNA;

3. Claims: 17-complete, 18-partially

A method for amplifying DNA using a thermostable enzyme exhibiting 3'-exonuclease-activity which enzyme is not or only to a negligible extend active on linear single stranded DNA;

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